# Beneficial substrate partitioning boosts non-aqueous catalysis in de novo enzyme-alginate beads

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**ABSTRACT:** Synthetic reactions often involve solvents incompatible with biocatalysts. Here, we encapsulate *de novo* heme-containing enzymes in calcium-alginate beads to facilitate heterogeneous biocatalysis in organic solvents. After encapsulation, enzymes remained structured and retained activity even when the beads are suspended in organic solvents. Carbene transferase activity, brought about by the heme cofactor, was enhanced when reactions were performed in organic solvent with alginate-encapsulated enzymes. Activity-solvent dependencies revealed that the activity boost is due to beneficial partitioning of the substrate between the beads and organic phase. Encapsulation furthermore facilitates enzyme recycling after the reaction. Alginate encapsulation opens up novel opportunities for biocatalysis in organic solvent systems, combining desired solvent properties of organic chemistry with enzymatic selectivity and proficiency.

Catalysis is key to the economic and sustainable synthesis of industrial chemicals.[1] Growing concerns over feedstock security, rising energy costs, and sustainability have amplified demands for greener catalysts. To satisfy that demand, biocatalysis has emerged as an attractive alternative over traditional and often toxic inorganic catalysts. The biocatalytic toolbox is constantly expanded by discovering natural enzymes,[2] *de novo* biocatalysts creation,[3] and directed evolution of promiscuous activities.[4] Bioengineering has afforded catalysts with tailored selectivities, accepting non-natural substrates, and even catalyzing abiological transformations.[4]

Although tailor-made enzymes can target countless transformations, typical organic reaction conditions are often detrimental to activity.[5] Though some proteins such as lipases and selected extremophilic enzymes retain activity in organic solvents,[6] most enzymes exhibit poor catalytic activities as they are either unstable or insoluble in non-aqueous conditions.[7] From a biotechnological perspective, the advantages of non-aqueous enzymology include i) increased solubility of organic compounds, ii) suppression of unwanted side reactions, iii) ease of product extraction, and iv) enzyme recoverability. Protein immobilization can shield enzymes from solvent-induced denaturation by providing a protective aqueous layer.[8] Protein encapsulation in calcium-alginate gels is provides one way to exploit this principle for organic enzymology.[9] Alginate is a polysaccharide in the cell walls of brown algae that instantaneously forms gelatinous water-insoluble beads upon dropwise addition to aqueous $CaCl_2$ solutions. This approach has proven advantageous in the past, giving rise to potent biotechnological applications including flow-reactors and enzyme recycling.[9e-i] However, enzyme encapsulation often decreases production yield compared to free enzyme.[9c, 9d]

Here, we set out to analyze the interplay of alginate encapsulation and activity in organic solvents. Such experiments would ideally rely on a single catalyst that allows exploring multiple reactions. We recently created C45, a *de novo* thermostable and catalytically promiscuous α-helical heme-containing enzyme. C45 is a proficient peroxidase[10] that can also act as a highly selective carbene transferase, catalyzing cyclopropanation, N-H insertion, carbonyl olefination, and homologous ring expansion reactions.[11] Here, we show that enzyme immobilization in calcium-alginate beads profit from heterogenous catalysis in organic solvents owing to beneficial partition of the substrates between beads and solvent (Fig. 1). This provides a general approach to perform proficient and selective biocatalysis in non-aqueous solvent systems.

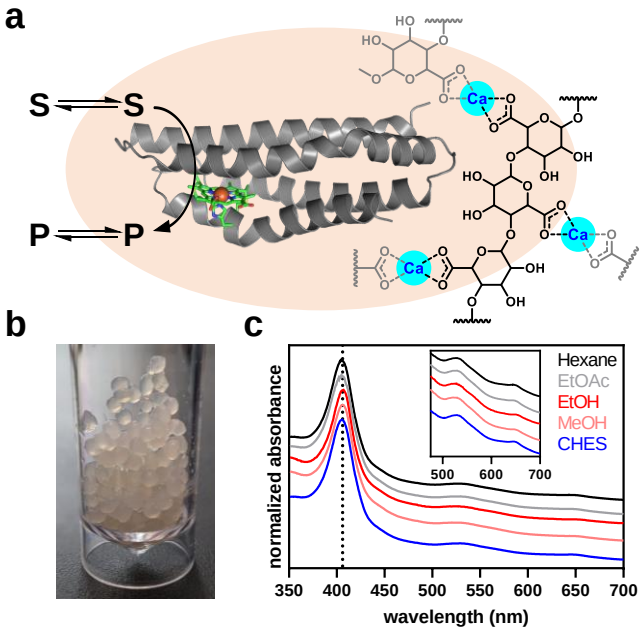



**Figure 1: Enzyme encapsulation in calcium-alginate beads. (a+b)** C45 is encapsulated in alginate by dropping an enzyme-alginate solution into aqueous calcium chloride. **(c)** Suspension of alginate gels in different solvents does not affect the heme absorbance spectrum.

Enzymes are readily immobilized in calcium-alginate gels by dropwise addition of a protein-alginate solution into aqueous calcium chloride (Fig. 1a).[12] Instantaneous gelation is based on ionic interactions of calcium with the alginate carboxylate groups.[13] Alginate cross-linking allows immobilization but also reduces the free volume available

inside the gel.[14] As expected, leakage decreased with increasing calcium chloride.[14-15] An ideal trade-off was observed for alginate beads formed with 300 mM $Ca^{2+}$ and 3% alginate (Tab. S1). The resulting beads had a volume of 14 µl and excellently retained a range of heme-proteins, as shown by the absorbance of heme leaking into solution. The beads' water content indicates a significant free volume to accommodate substrates and products (Tab. S1). The characteristic Soret peak (406 nm) and Q bands (530 nm and 650 nm) absorbances of the heme cofactor in C45 provide insights into its structural integrity. The spectra of alginate-encapsulated C45 (AE-C45) suspended in various organic are virtually identical to the spectrum of free C45 in buffer (Fig. 1). Similar observations were made with cytochrome *c*, a heme containing natural protein (Fig. S1). Alginate encapsulation is thus a general and mild method protecting the encapsulated enzymes from solvent denaturation.

Carbene transfer reaction proceeds via an iron(II) carbene intermediate formed by mixing EDA with ferrous C45.[11] Characteristic changes in the heme spectra can be used to follow heme reduction and carbene formation in the beads. Sodium dithionite was used to reduce the heme-cofactor to its ferrous form and was monitored by a redshift of the Soret peak to 421 nm and Q-bands at 520 nm and 550 nm. Notably, heme reduction inside the beads is much slower than with free enzyme (Fig. 2a). This indicates a kinetic barrier separating the beads and bulk solvent. For reduction with dithionite, which practically happens instantaneously, diffusion into the bead is apparently rate limiting. After reduction, EDA can be added leading to carbene formation, which is detected by a further shift of the Soret peak to 426 nm and Q-bands to 542 nm and 584 nm (Fig. 2b). Reaction of EDA with free and encapsulated C45 resulted in identical spectra (Fig. 2c), confirming the formation of the same carbene-intermediate. It so noted that carbene formation is much slower than heme reduction, and proceeded with a maximum rate of 0.5 $s^{-1}$ as previously determined.[11a] For carbene formation, as well as later carbene transfer, equilibrium partition between solvent and bead will likely outweigh diffusion in determining the overall reaction kinetics and yield.

Having demonstrated that alginate-encapsulated C45 forms a metallocarbenoid intermediate, we next explored its carbene transferase activity by N-H insertions (Scheme 1). N-H insertion into piperidine by encapsulated C45 provided ethyl-1-piperidine acetate with a yield of 14% as determined by HPLC (Fig. 2d, Tab. S2). Similar to previous observations for alginate encapsulated enzymes, the yield decreased compared to free enzyme. Notably, yields could be drastically boosted by adjusting the solvent system. The highest yields of 96-98% were obtained in apolar solvents. Under these conditions performance excels that of free enzyme, which requires a 20-fold higher catalyst loading to achieve a 79% yield.[11a]

Activity-solvent dependencies reveal the origins of the observed activity gain: Decreasing solvent polarity increased the yield. To further dissect this trend, we determined partition and diffusion constants of the piperidine substrate into the beads ($\kappa_{pip}$ and $D_{pip}$). To that end, empty beads were stirred in various solvents containing piperidine and the decrease in piperidine concentration in the liquid phase was determined to calculate $\kappa_{pip}$ and $D_{pip}$. The calculated $D_{pip}$ correlated well with N-H insertion yield (Fig. 2d). This shows that the solvent strongly affects the effective substrate concentration inside the bead. The slow reduction of heme inside the beads suggests that both the ensuing carbene formation and N-H insertion will be limited by substrate diffusion into the beads. Our data suggest a kinetic effect, but we cannot exclude that equilibrium partition will be determining to the overall yield. Nonetheless, both a kinetic and equilibrium interpretation imply that a change in effective substrate concentration is key to determine activity. This trends can be exploited by choosing the ideal solvent system for the desired transformation to boost product yield.

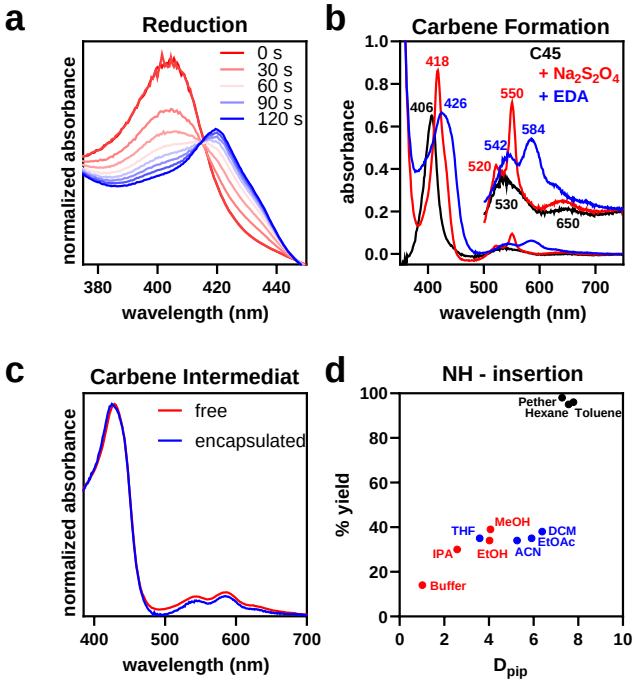
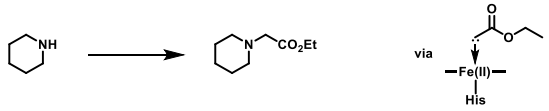



**Figure 2: Carbene transfer activity of encapsulated C45 in organic solvent. (a)** Slow reduction of C45 by dithionite indicates diffusion barrier across the bead surface. **(b)** Reduction and carbene formation of encapsulated C45 indicated by characteristic spectroscopic shifts. **(c)** Carbene-formation results in spectroscopically identical intermediates for free and encapsulated C45. **(d)** Product yields for N-H insertion into piperidine correlate with the substrate diffusion coefficients, indicating that yields are improved by increasing the effective substrate concentration inside the beads.

**Scheme 1. C45 mediated NH-insertion via carbene-transfer**




The effect of partition was furthermore probed for peroxidase activity. Based on our previous observations, it is expected that addition of organic solvent will increase the apparent peroxide concentration in the bead. Given that high concentrations of peroxide often have deleterious effects on enzyme activity, encapsulation should result in significant activity losses. Peroxidase activity of alginate-encapsulated C45 was analyzed by monitoring oxidation of guaiacol by

hydrogen peroxide (Fig. 3 and Scheme 2). Encapsulated C45 retained activity with a $k_{cat}$ of 170 $s^{-1}$ under limiting guaiacol concentration (1 mM), which is only 11-fold lower than what was observed for free C45. The apparent $K_m$ decreased 4.5-fold upon encapsulation, signaling an increased apparent peroxide concentration inside the beads, which likely also causes the apparent drop in $k_{cat}$. Switching the solvent system to any other organic solvents only further decreased activity, which signals an even higher effective peroxide concentration inside the beads due to partitioning between the organic and aqueous phases. Similar observations were made with horseradish peroxidase, which retained some activity after encapsulation, as well as when using ABTS as a different peroxidase substrate (Fig. S3). The effects on peroxidase activity support our model, and increase apparent substrate concentration by partitioning. Our model may explain the activity losses observed for other systems,[9c, 9d] and suggest opportunities to exploit substrate partitioning to boost enzyme activity in specific cases.

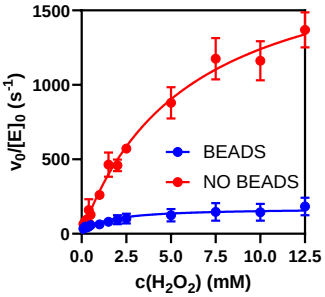
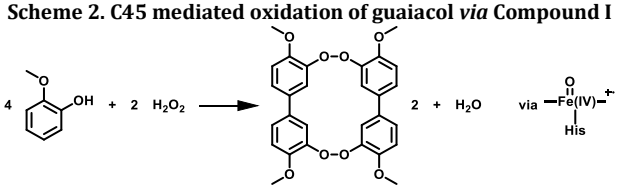



**Figure 3: Peroxidase activity.** C45 encapsulated in calcium-alginate beads retained activity for guiacol oxidation. Activity is decreased, likely due to non-beneficial partitioning of peroxide into the beads.

**Scheme 2. C45 mediated oxidation of guaiacol *via* Compound I**




Enzyme recyclability is highly desirable from an industrial perspective, and alginate-beads can readily be recovered and reused. To test the reusability of encapsulate C45, beads were first employed to catalyze the cyclopropanation of styrene, (Fig. 4, Tab. S3 and Scheme 3). After removal of the reaction mixture, beads were reused to catalyze carbene N-H insertion into piperidine. Cyclopropanation proceeded with moderately high yields (62-79%) and enantioselectivity (86-99%). The observed product enantioselectivity confirms that C45 remains active and folded after encapsulation. After isolating, washing, and re-deploying the C45 beads used in the cyclopropanation reaction the N-H insertion product can be readily detected although with 30-65% residual activity. No cyclopropanation product was detected in any of the N-H insertion reaction chromatograms. The absence of cross contamination with residual reagents as well as formation of different product during the second reaction clearly confirms that the encapsulated C45 can be recovered and remains active towards carbene transferase chemistry in successive reactions.

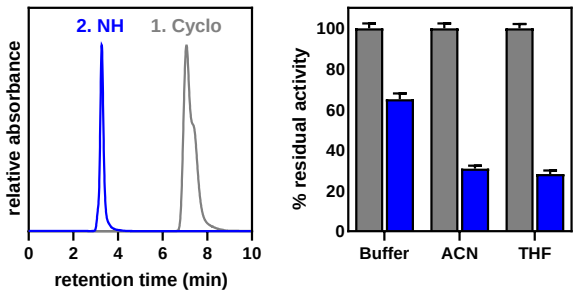
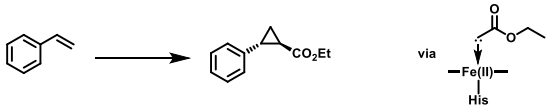



**Figure 4: Encapsulated enzymes can be readily recycled.** Encapsulated C45 has been employed to sequentially promote cyclopropanation (1. Cyclo, gray) followed by NH-insertion reaction (2. NH, blue) with some losses in activity.

**Scheme 3. C45 mediated cyclopropanation via carbene-transfer**




Enzyme encapsulation in alginate beads allows to perform biocatalysis under conditions that are detrimental to the free enzyme. The beads introduce a diffusion barrier, that leads is rate limiting for fast reactions such as peroxidase activity and carbene formation. The effective substrate concentration inside the bead is key to activity and can be manipulated by choosing the ideal solvent system. Recoverability and recyclability are added application benefits of alginate-encapsulation. Enzyme immobilization in alginate beads is a versatile method and this work provides a rational basis to tune solvent systems to maximize product yields.

## ASSOCIATED CONTENT

### Supporting Information

Supporting Information contain complete experimental procedures and additional kinetic data, including Table S1-S3 and Figures S1–S3.

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### Author Contributions

R.S. and H.A.B. contributed equally. R.S., H.A.B., and J.L.R.A. designed the experiments. R.S. and H.A.B. performed the experiments. R.S., H.A.B., and J.L.R.A. wrote the manuscript with input from A.J.M.

### Notes

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

This work was supported by the Biological and Biotechnological Sciences Research Council (BBI014063/1, BB/R016445/1, and BB/M025624/1), the Engineering and Physical Sciences Research Council (EP/R026939/1, EP/R027129/1 and EP/M022609/1) and the Bristol Centre for Functional Nanomaterials (Engineering and Physical Sciences Research Council Doctoral Training Centre Grant EP/G036780/1) through a studentship for RS. HAB thanks the Swiss National Science Foundation for a Postdoc.Mobility fellowship.

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